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T Cells Can Be Activated by Peptides That Are Unrelated in Sequence to Their Selecting Peptide

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Summary

We tested the ability of CD4$^+$ T cells, selected in the thymus by reaction with class II protein bound to a single peptide, to react with the same class II protein bound to other peptides. The T cells reacted with all peptides tested, including one that was quite unlike the selecting peptide in T cell receptor binding residues. The receptors on class II/peptide–reactive T cells from class II/single peptide mice were similar but not identical to some of those from normal animals. Thus, class II bound to a single peptide selects a subset of T cells that is related to that selected by class II bound to many peptides.

Introduction

Thymocyte positive selection was first discovered because T cells that mature in thymi expressing major histocompatibility complex (MHC) proteins of one allele are able to respond to foreign peptides bound to MHC proteins of that allele, but not to foreign peptides bound to MHC proteins of a different allele (Bevan, 1977; Zinkernagel et al., 1978). This observation led to the idea that thymocytes bearing $\alpha\beta$ T cell antigen receptors (TCRs) mature only if their TCRs have some appreciable affinity for MHC proteins they encounter in the thymus, the process now called positive selection. The discovery of negative selection in the thymus led to modifications of this idea, and subsequent experiments have now demonstrated that thymocytes mature only if their TCRs have some appreciable but low avidity for thymic MHC molecules (Lo et al., 1986; Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994). Reactions between TCRs on thymocytes and MHC proteins that are of too high avidity lead to death of the thymocyte (Kappler et al., 1987).

Hypotheses about positive selection have also been modified by the discovery that on normal cells, MHC proteins are bound to many different peptides from the host (Falk et al., 1991; Rudensky et al., 1991a; Marrack et al., 1993). It is now thought that positive selection of thymocytes bearing a particular TCR involves reaction between that TCR and not only the MHC protein but also the peptide engaged by that MHC (Hogquist et al., 1994; Ashton-Rickardt et al., 1994; Sebzda et al., 1994). This realization has raised questions about the relationship between the sequence of the peptide involved in positive selection and that involved in activation of a particular cell. Also there is the matter of the degeneracy of the phenomenon, since a single MHC allele can select T cells bearing millions of different TCRs. How limited is the TCR repertoire if only a single MHC protein bound to a single peptide is involved?

Recent advances have revealed some answers to these questions. For example, early experiments suggested that the selecting peptide might need to be related in sequence to the subsequently activating peptide for a particular T cell (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994). Later experiments contradicted this idea, however. In particular, Pawlowski et al. (1996) showed that thymocytes bearing a transgenic TCR could be positively selected by peptides that were unrelated to the peptide that could activate T cells bearing the same TCR. Other experiments have shown that a single MHC/peptide combination selects an unexpectedly large number of T cells, suggesting that the specificity during positive selection of TCRs for peptide might not be especially tight (Ignatowicz et al., 1996; Martin et al., 1996; Miyazaki et al., 1996).

In this report we describe experiments that address the latter question with the use of mice that express a single class II MHC protein bound to a single peptide. T cells selected on this combination were tested for their ability to respond to various peptide antigens, some of which were relatively unrelated in their TCR contact residues to the selecting peptide. T cells selected on the MHC/single peptide combination responded to all peptides tested. The sequences of the TCRs on these selected cells were related to but not identical to those of T cells from normal mice, specific for the same MHC/peptide combination. We conclude that the reaction between TCRs and MHC/peptide during positive selection does not necessarily dictate the peptide specificity of the selected, mature T cell. Moreover, MHC bound to a single peptide can select T cells specific for many different peptide ligands.

Results

We have previously described mice that express a transgene coding for IAβ^b covalently bound to a peptide from Eα, Eα 52–68 (Ep) (Ignatowicz et al., 1996). The transgene was crossed into mice that lacked functional

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Table 1. T Cells Selected by a Single MHC/Peptide Ligand Can Respond to the Same MHC Bound to Many Peptides

Peptide		Number of A^bEpli^- T Cells[a] Responding to:	
Source	Sequence[b]	A^b/Peptide > A^bwtli$^-$	A^b/Peptide Only
Ep (Eα 52–66)	ASFEAQGALANIAVDKA	—	—
Ep60K	FEAQGAKANIAVD	2	1
Ep3K	FEAQKAKANKAVD	4	3
Ep3E	FEAQEAEANEAVD	1	0
IgGVH 59–74	NADFKTPATLTVD	1	1
Unknown	NYNAYNATPATLAVD	6	2
PCC 43–58	AEGFSYTDANKNKGIT	10	6

[a] T cells were prepared as T cell hybridomas. Numbers refer to the number of independent T cell hybridomas with the indicated specificity obtained from a single in vitro peptide priming experiment for each peptide except PCC. For PCC the numbers represent the number of T cell hybridomas with the indicated specificity from three in vitro priming attempts and one in vivo priming experiment.
[b] Peptides are listed so that their registers when bound to A^b are aligned. Alignments were established as described in this report and elsewhere (Ogasawara et al., 1987; Fremont et al., 1996; D. H. Fremont and E. R. Unanue, unpublished data). The peptide amino acids that are predicted to be most likely to contact TCRs are underlined. Reactive T cells were evaluated as T cell hybridomas after peptide priming and creation of hybrids.

Aβ genes (Grusby et al., 1991) and that also lacked invariant chain (Ii) (Bikoff et al., 1993). Mice were thus produced in which all expressed class II proteins were of a single MHC allele, A^b, bound to a single peptide, Ep (A^bEpIi^-). As we have previously shown, absence of Ii was essential for this because, in the presence of Ii, the A^bEp complex was chaperoned through the endosomal compartment en route to the cell surface (Lamb and Cresswell, 1992; Elliott et al., 1994). In this compartment the covalently bound peptide was removed from the A^b molecule and replaced by other peptides (Ignatowicz et al., 1995; Ignatowicz et al., 1996).

$CD4^+$ T cells are selected in the thymi of these A^bEpIi^- animals. Many of these selected $CD4^+$ T cells react with A^b bound to the many mouse derived peptides with which it is associated in wild-type mice (A^bwt) (Ignatowicz et al. , 1996). Also, the selected $CD4^+$ T cells bear TCRs that include all of the available mouse Vβs, with approximately the same frequency as in A^bwt animals.

The experiments reported here were designed to investigate the TCR repertoires of T cells from the A^bEpIi^- mice in more detail. To do this we needed to prepare peptides that could bind to A^b and be used as immunogens for $CD4^+$ A^bEpIi^- T cells (as described below).

Studies on Peptide Binding to A^b

Peptide binding to A^b has been studied in detail by several groups (Ogasawara et al., 1987; Itoh et al., 1992; Wall et al., 1994). However, the structure of A^b bound to a peptide has not been solved by x-ray crystallography, and the published studies disagree about which amino acids of which peptides bind to the groove of A^b. The amino acid sequences of some peptides known to bind to A^b are shown in Table 1.

To establish the frame with which they bind to A^b, truncated forms of some of these peptides were prepared and assayed for their ability to bind to cell surface A^b. Early studies, involving stimulation of T cell hybridomas specific for A^bEp (Ignatowicz et al., 1995), showed that three amino acids could be removed from the C-terminal end of Ep without affecting its activity. Removal of an additional two amino acids, however, made the peptide inactive (data not shown).

A second assay used an antibody that reacts with A^b engaged to Ep, anti-A^bEp (Murphy et al., 1989; Rudensky et al., 1991b). This antibody was used in cytofluorometric studies to measure the binding of Ep lacking two C-terminal amino acids, Eα 52–66, and N-terminal truncations of this peptide to A^b on spleen cells from A^bwtli$^-$ mice. On these cells A^b is engaged by peptides that do not bind strongly to the protein and that can be readily displaced by other peptides (Bikoff et al., 1993; Bodmer et al., 1994). The data in Figure 1A show that four amino acids could be removed from the N-terminal end of Eα 52–66 without affecting its ability to bind to A^b. Removal of the fifth amino acid, A, from its N terminus reduced binding, and removal of the sixth amino acid, Q, completely abolished binding (Figure 1A). It is possible that this result was due to failure to detect Eα 57–66 bound to A^b with the anti-A^bEp antibody. However, we do not think this is so, since Eα 58–66 did not compete with Ep (that is, Eα 52–68) for binding to A^b (data not shown).

Collectively these results show that the minimum sequence of Ep needed to bind to A^b is Eα 57–65.

Data from the known structure of peptides bound to IE^k and preliminary data from the structure of IA^k bound to a peptide suggest that peptide binding to class II proteins involves four peptide-binding pockets, at amino acid positions P1, P4, P6, and P9 in the peptide (Fremont et al., 1996; D. Fremont and E. Unanue, unpublished data). Since engagement of Ep by A^b involves amino acids 57–65 of the peptide, Q57, L60, N62, and V65 are probably buried in the groove of A^b, as shown in Table 1. This is not the alignment that has been suggested by previous studies (Wall et al., 1994). However, we believe that the truncation experiments reported here are definitive and therefore that they indicate the correct alignment.

Similar experiments were done using truncations of PCC 43–58 (PCC), a peptide shown by Suzuki and Schwartz (1986) to bind A^b. A short version of this peptide, PCC 43–54, stimulated a T cell specific for A^b/PCC. Other shortened peptides, PCC 45–56 and PCC 47–58, did not. We concluded that the alignment of this peptide bound to A^b is as shown in Table 1 with anchor residues A43, F46, Y48, and A51.

A third assay was used to assess the binding to A^b of some of the other peptides shown in Table 1. In the

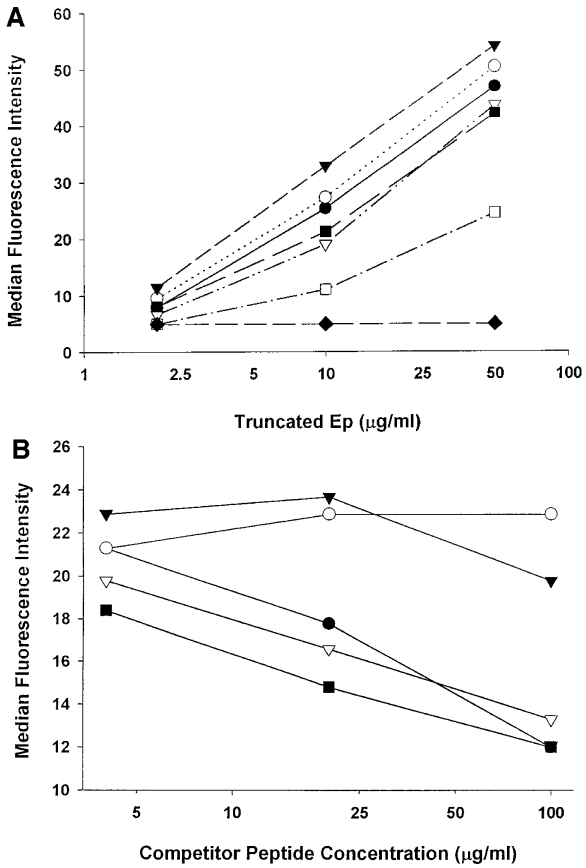

Figure 1. Assessment of Peptide Binding to A^b

(A) Eα57–66 is sufficient for binding of the peptide to A^b. Truncated versions of Ep (Eα 52–68) were incubated with A^bwtli$^-$ spleen cells and their binding assessed using anti-A^bEp antibody as described in Experimental Procedures. Data shown are the median fluorescence intensities of the cells after incubation with the indicated concentrations of the peptides and staining with biotinylated anti-A^bEp followed by phycoerythrin-coupled streptavidin. The peptides used were as follows: filled circles, Eα 52–66; open circles, Eα 53–66; filled triangles, Eα 54–66; open triangles, Eα 55–66; filled squares, Eα 56–66; open squares, Eα 57–66; and filled diamonds, Eα 58–66.

(B) Assessment of the ability of various peptides to bind to A^b using a competitor assay. Spleen cells from A^bwtli$^-$ mice were incubated with a limiting amount of Eα 52–66 and various concentrations of other peptides thought to bind A^b. Binding of Eα 52–66 was assessed using the anti-A^bEp antibody as described above and in Experimental Procedures. The peptides used were as follows: filled squares, unknown; open triangles, IgGVH 59–74; filled circles, Ep3K; filled triangles, Ep3E; and open circles, PCC. The sequences of these peptides are shown in Table 1.

assay the ability of the peptides to compete with Ep for binding to A^b was measured using anti-A^bEp as an indicator. As shown in Figure 1B, peptides Unknown, IgGVH, and Ep3K inhibited Ep binding to A^b well. Therefore all of these peptides probably bind strongly to A^b. At high concentration Ep3E inhibited Ep binding to A^b somewhat, and therefore this peptide probably has poor but appreciable affinity for A^b. The PCC 43–58 peptide did not compete with Ep for binding to A^b at even the highest concentration tested. This peptide probably binds A^b poorly, perhaps because some of its anchor residues are not ideal for binding to this class II protein (Table 1). However, several prior studies (Ogasawara et al., 1987; Suzuki and Schwartz, 1986) and those reported in this article show that this peptide in combination with A^b is able to stimulate T cells; therefore it must have some affinity for this class II protein.

The probable registers of these peptides when bound to A^b are indicated in Table 1. Assignments of these registers are supported by the Ep binding data described above, by the finding that a version of the Unknown peptide truncated at its N-terminal end by four amino acids binds to A^b as well as does the full-length Unknown peptide and by the results with the PCC truncated peptides. The L60K substitution in Ep60K and Ep3K did not affect binding of these peptides, even though this amino acid is predicted to be buried in an A^b pocket. If the structure of A^b is like that of IA^k, the pocket at P4 will be large and able to accommodate many amino acids.

Isolation of Peptide-Specific T Cells from A^bEpli$^-$ Mice

In the past we were not able to examine the ability of CD4$^+$ T cells from A^bEpli$^-$ mice to react with foreign peptides bound to A^b because we were not able to prime the animals directly with peptide. Since all of the grooves of the class II proteins in these animals are occupied with the covalent Ep, exogenous peptides cannot enter these grooves and prime T cells (L. I., unpublished data).

Two strategies were used to circumvent this problem. In the first, CD4$^+$ T cells from A^bEpli$^-$ animals were purified and cultured with antigen-presenting cells from A^bwtli$^-$ animals and high concentrations of a foreign peptide known to bind A^b. Controls involved CD4$^+$ T cells from class II$^-$ Ii$^-$ (C2$^-$) mice (Ignatowicz et al., 1996). Four to five days later, activated T cells were harvested from these cultures, expanded for 3 days, and converted into T cell hybridomas by fusion with BWα$^-$β$^-$ (White et al., 1989). The hybrids were then assayed for their ability to respond to spleen cells from A^bwtli$^-$ mice in the presence or absence of the immunizing peptide or a control peptide that could also bind to A^b. Some examples of the specificities of the hybridomas thus created are shown in Table 2.

Almost all of the hybridomas responded to A^bwtli$^-$ spleen cells whether or not the immunizing peptide was present. These are exemplified by 60K-39, 3K-46, and PCC-13 in Table 2. We have previously shown that T cells in A^bEpli$^-$ mice are not tolerant to A^b bound to the peptides with which it is engaged in wild-type or Ii$^-$ mice (Ignatowicz et al., 1996). Thus we believe that the T cell parents of these hybrids must have responded to A^b on the antigen-presenting cells occupied by one of the (relatively few) mouse peptides with which A^b is engaged in Ii$^-$ animals (Bodmer et al., 1994). These hybridomas were excluded from further study.

For each peptide immunogen a few of the hybridomas responded to A^bwtli$^-$ cells in the presence of a control peptide but responded to the same cells much more strongly when the immunizing peptide was added. These are exemplified by 60K-32, 3K-14, and PCC-220 in Table 2. Other hybridomas (3K-30 and PCC-31, Table

Table 2. Reactivities of Hybridomas Prepared from A^bEpli^- T Cells Primed with Peptides In Vitro

Hybridoma	Priming Peptide[a]	Response (units/ml IL-2 Secreted) in the Presence of Ii^- Spleen Cells plus: Control Peptide[b]	Priming Peptide
60K-39	Ep60K	138	145
60K-32	Ep60K	111	815
3K-46	Ep3K	167	240
3K-14	Ep3K	53	1262
3K-30	Ep3K	<1	255
PCC-13	PCC 43–58	422	478
PCC-220	PCC 43–58	3	76
PCC-31	PCC 43–58	<1	4363

[a] Peptide sequences are given in Table 1.
[b] Control peptides were Ep60K or PCC 43–58.

2) did not respond at all to A^bwtIi^- cells in the absence of the immunizing peptide but responded strongly when the peptide was added. Both of these groups of hybridomas were considered to be specific for A^b bound to the immunizing peptide.

Peptides Ep60K, Ep3K, and Ep3E were designed before we knew the register of Ep bound to A^b. However, after the register problem was solved, we found that the only amino acid that differed between Ep and Ep60K is probably buried in the P4 pocket of A^b. Nevertheless, T cells specific for A^b bound to Ep60K and nonreactive with A^bEp were generated from A^bEpIi^- T cells. Other studies have shown that peptide amino acids that are buried in MHC grooves can affect T cell reactivity by slightly changing the exposed face of the MHC/peptide combination (Evavold et al., 1993; Fremont et al., 1996). Probably such effects account for the reactivities seen here.

One A^b/PCC–specific T cell hybridoma was made in a different way. In this method chimeras were made by reconstituting lethally irradiated A^bEpIi^- mice with A^bwtIi^+ fetal liver. After recovery, such animals contained $CD4^+$ T cells that had been positively selected on A^bEp as well as antigen-presenting cells bearing A^bwt. The $CD4^+$ T cell repertoire in these mice was only a subset of that in unmanipulated A^bEpIi^- mice, because the $CD4^+$ T cells in the chimeras should be tolerant to A^b bound to its normal collection of mouse peptides (L. I. and P. M., unpublished data).

Two chimeric and two A^bwtIi^+ mice were primed with the PCC peptide. Three A^b/PCC–specific T cell hybridomas were obtained from the chimeric animals. However, these were found to have identical sequences for their TCR α and β chains, so we concluded that they were the products of independent fusions to cells of the same expanded T cell clone. Many A^b/PCC–specific hybrids with many different TCRs were obtained from the A^bwtIi^+ fusion. These results allowed us to compare the magnitude of the TCR repertoires of T cells tolerant to the same spectrum of class II and self peptides, but positively selected on MHC bound to one or many peptides. The results indicate that the MHC/single peptide–selected repertoire is probably much smaller than the MHC/many peptide repertoire.

The Activating Peptide Is Not Necessarily Related in TCR Contact Residues to the Selecting Peptide

Peptides shown to bind A^b (Table 1) were used in vitro to prime T cells from A^bEpIi^- mice and converted into T cell hybridomas. T cells specific for A^b bound to each of these peptides were generated (Table 1). For most peptides, one or more of the T cells showed no detectable reactivity for A^b in the presence of a control peptide and responded well when the priming peptide was added. For all of the peptides, some T cells were generated that responded well to A^b plus the priming peptide and also cross-reacted weakly with A^b bound to mouse peptides. Hence, T cells from A^bEpIi^- mice could react specifically with A^b bound to each of the peptides shown in Table 1, except Ep, which was not tested. These reactivities were due to recognition of A^b bound to the priming peptide and not to the higher levels of A^b induced on A^bwtIi^- cells by A^b binding peptides (Bikoff et al., 1993).

The amino acids of the peptides that were predicted to engage TCRs were not always similar to those of Ep. For example, the residues predicted to be at P2, P3, P5, P7, and P8 are G, A, A, I, and A for Ep, but are K, A, A, K, and A for Ep3K and are E, G, S, T, and D for PCC.

From these results we concluded that the peptides involved in positive selection or activation of particular T cells need not be closely related to each other in TCR contact residues. This conclusion has been reached by some but not all investigators in the field (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994, Pawlowski et al., 1996; Nakano et al., 1997).

The TCRs on T Cells Selected on A^bEp Are Related to Those Selected on A^b Bound to Many Peptides

We wished to compare the TCRs on peptide-specific T cells selected on A^bEp with those on T cells selected in A^bwt animals. T cells specific for A^b/PCC were chosen for these experiments because it is already well known that A^bwt animals respond well to this peptide. A^bwt mice were primed with PCC, and T cell hybridomas were generated. Two primed A^bwt mice generated at least 100 A^b/PCC–specific T cell hybridomas. None of the more than 20 examined in detail had the same TCR as

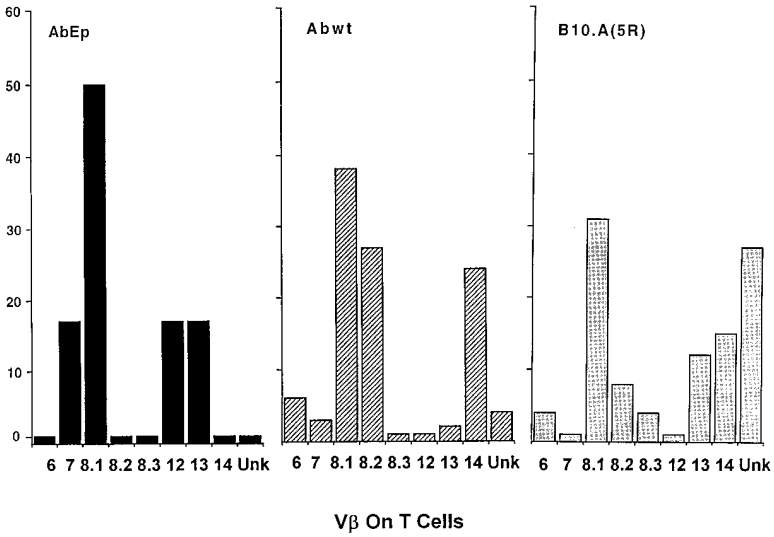

Figure 2. The Vβ Repertoire of Peptide-Specific TCRs on A^bEpli^- T Cells Is Related to That on A^bwt T Cells

T cell hybridomas specific for A^b/PCC were prepared from A^bEpli^-, A^bwt, and B10.A(5R) mice. The Vβs they bore were identified by staining with anti-Vβ antibodies. The percentages shown in the Figure were obtained from 5 hybridomas from A^bEpli^- mice combined with 1 hybridoma from A^bEpli^- chimeric animals, 90 hybridomas from A^bwt animals, and 26 hybridomas from B10.A(5R) mice. Only the Vβs for which at least 1 positive hybridoma was found are shown. Unknown (Unk) indicates Vβs for which antibodies are not currently available but which are expressed in these mice, that is, Vβs 1, 15, 16, 18, or 20.

any other; therefore the TCR repertoire of A^bwt mice for this A^b/peptide combination is large.

Even so, there were some restrictions on the variability of these A^bwt TCRs. As shown in Figure 2, most of the A^b/PCC TCRs from A^bwt mice bore one of four Vβs: Vβs 6, 8.1, 8.2, or 14. The Vβ8.1-bearing TCRs included several different Jβs, of which Jβ2.6 was the most common (Figure 3). The A^bwt hybridomas bearing Vβ8.1 and Jβ2.6 tended to use members of the Vα10 family (Figure 4). These α chains also used Jαs with a K two amino acids N-terminal to the conserved Jα FG sequence. This basic amino acid is present in about 70% of Jαs. The CDR3 regions of the α chains were rich in short uncharged hydrophilic amino acids such as N, S, and T.

T cells from A^bEpli^- mice were primed with PCC as described above. Six A^b/PCC–specific hybridomas were obtained from three in vitro priming attempts and one in vivo chimera experiment. None of these bore Vβs 6, 8.2, and 14, although these Vβs were used by many of the A^b/PCC–specific T cells from A^bwt mice. However, three of the A^bEp-selected hybridomas used Vβ8.1 in their TCRs (Figure 2), the Vβ used most commonly by A^bwt-selected T cells with the same specificity. These three Vβ8.1-bearing A^bEp-selected hybrids used Jβ2.6 (Figure 3). Again, this was the Jβ used most commonly by A^bwt-selected T cells. The three A^bEp-selected Vβ8.1-bearing hybridomas did not bear α chains of the Vα 10 family, however. Instead, two of them used members of the Vα4 family (Figure 4). Vαs 4 and 10 are not particularly closely related to each other (Arden et al., 1995). Like the A^bwt Vβ8.1/Jβ6-bearing TCRs, those from A^bEpli^- mice had a basic amino acid in Jα two amino acids N-terminal to the conserved FG sequence. Their CDR3 regions were also rich in short hydrophilic amino acids.

Comparison of the D and N region sequences of the Vβ8.1/Jβ2.6– expressing β chains of TCRs specific for A^b bound to PCC revealed no marked similarities. The D and N region sequences of A^b/PCC-specific β chains selected on A^bEp or A^bwt were not particularly alike within or between the two groups.

It was likely that the TCR repertoire for A^b/PCC of T cells from A^bEpli^- mice differed from that of T cells from A^bwt animals because the MHC/single peptide combination did not positively select T cells bearing as many different TCRs as MHC bound to many peptides did. It was, however, formally possible that the difference might be due to some unexpected ability of A^b bound to Ep to tolerize T cells destined to be activated by A^b bound to PCC. To check this, the TCR repertoire for A^b/PCC of T cells from B10.A(5R) mice was measured. B10.A(5R) animals express A^b/Ep, and A^b bound to the many peptides with which it is engaged in A^bwt mice (Murphy et al., 1989). Many A^b/PCC–specific T cells were obtained from 2 B10.A(5R) animals primed with PCC. These hybridomas bore various Vβs with approximately the same distribution as those from A^bwt mice (Figure

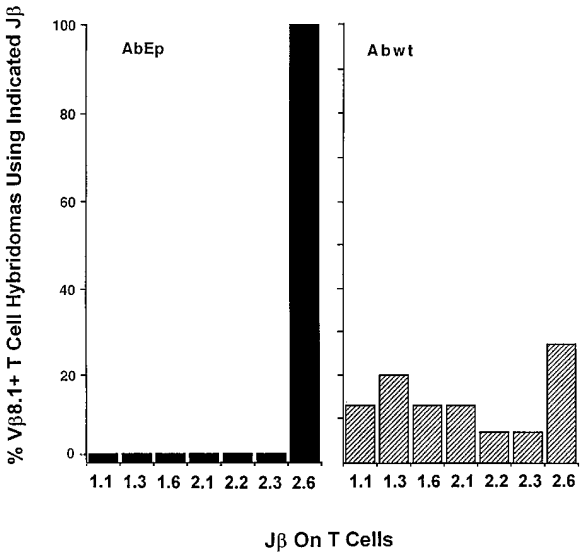

Figure 3. The Jβ Repertoire of Peptide-Specific TCRs on A^bEpli^- T Cells Is Related to That on A^bwt T Cells

The β chains of Vβ8.1 using TCRs from A^bEpli^- or A^bwt mice were sequenced and the Jβs they used thus identified. The percentages shown were obtained from 3 Vβ8.1 using TCRs from A^bEpli^- mice and from 15 Vβ8.1 using TCRs from A^bwt mice.

A

Donor	T cell	Vα	CDR3 Sequence	Jα
A^bEp	IrTgPCC-2.1	mAV4S2	TGTGCTTTGAGA**GCGAGT**ACAAATGCTTACAAAGTCATCTTTGGA	mAJ23
			C A L R **A T** T N A Y K V I F G	
A^bEp	TgPCC-5	mAV2S8	TGTGCAGCAAG**GTCT**AATACAGGAAAC**T**ACAAATACGTCTTTGGA	mAJ33
			C A A **R S** N T G N Y K Y V F G	
A^bEp	TgPCC-123	mAV4S4	TGTGCACTGGGT **GGGGGGA**CCAGAATCTTCTTTGGT	mAJ24
			C A L G **G G T** R I F F G	
A^bwt	BP-1	mAV3S9	TGTGCTGTGAGCTCC **TC**GGGATACAACAAACTCACTTTTGGA	mAJ9p
			C A V S S **S** G Y N K L T F G	
A^bwt	BP-75	mAV10S7	TGTGCTATGAACAGTGGAGGCAGCAATTACAAACTGACATTTGGG	mAJ45
			C A M N S G G S N Y K L T F G	
A^bwt	H21	mAV10S1	TGTGCTTATTCT GGAGGAAGCAATGCAAAGCTAACCTTCGGG	mAJ34
			C A **Y** S G G S N A K L T F G	
A^bwt	H97	mAV10S1	TGTGCT**ATT**ACTAAC AGTGCAGGGAACAAGCTAACTTTTGGA	mAJ14
			C A **I** T N S A G N K L T F G	

B

Donor	T cell	CDR3 Sequence
A^bEp	IrTgPCC-2.1	TGTGCCAGCAGT**AAC** TGGGGGGGG**GT**ATGAACAGTACTTCGGT
		C A S S **N** W G G Y E Q Y F G
A^bEp	TgPCC-5	TGTGCCAGCAGTG**CCCCGGGACTGGGGGGC**TG**C**GAACAGTACTTCGGT
		C A S S **A P** G L G G **C** E Q Y F G
A^bEp	TgPCC-123	TGTGCCAGCAGACAG GTTTATGAACAGTACTTCGGT
		C A S R Q V Y E Q Y F G
A^bwt	BPCC-1	TGTGCCAGCAGGGGACAG GG**ACT**CTATGAACAGTA**CTT**CGGT
		C A S R G Q G **L** Y E Q Y F G
A^bwt	BPCC-75	TGTGCCAGCAGT**CCCGGGACTGGGGTGTTTT**ATGAACAGTACTTCGGT
		C A S S **P** G T G **V F** Y E Q Y F G
A^bwt	h21	TGTGCCAGCAGT**CCGGGACAG** CTC**T**ATGAACAGTACTTCGGT
		C A S S **P** G Q L **Y** E Q Y F G
A^bwt	H97	TGTGCCAGCAGTGAT**GCC**GGGACTGGCTCCTATGAACAGTACTTCGGT
		C A S S D **A** G T G S Y E Q Y F G

Figure 4. Sequences of A^b/PCC-Reactive Vβ8.1 and Jβ2.6 Using TCRs

The α and β chains of Vβ8.1 and Jβ2.6 using TCRs specific for A^b/PCC were obtained. Shown are three such sequences from T cells from A^bEpIi^- animals: IrTgPCC-2.1, TgPCC-5, and TgPCC-123. The IrTgPCC-2.1 T cell hybridoma was obtained from A^bEpIi^- chimeric mice, primed in vivo with the peptide. The other two hybridomas were obtained from in vitro priming experiments. Also shown are four sequences from T cells from A^bwt mice, primed in vivo. Bold characters indicate non–germline-encoded nucleotides and amino acids. (A) α chain sequences.
(B) β chain sequences.

2). Hence the restricted TCR repertoire of A^b/PCC–specific T cells from A^bEpIi^- mice was due to limited positive selection, not tolerance to A^bEp.

Discussion

Experiments with both class I and class II have shown that a single MHC/peptide combination caused positive selection of surprisingly large numbers of T cells (Ashton-Rickardt et al., 1993; Hogquist et al., 1993; Ignatowicz et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). However, it has only just become apparent that T cells specific for the relevant MHC molecule bound to many different peptides are included among such T cells. Not only are T cells selected on MHC bound to a single peptide activated by many other peptides, but also the activating peptides need not be related in TCR contact residues to the selecting peptide (Nakano et al., 1997; the present study). The latter finding is particularly surprising given some prior publications that showed that selection of T cells bearing a particular transgenic TCR required selecting peptides that were related in amino acid sequence to the activating peptides for T cells bearing those TCRs (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994; but see Pawlowski et al., 1996).

For T cell activation, TCRs must react with their MHC/peptide ligands with moderate affinity (Matsui et al., 1991; Weber et al., 1992; Alam et al., 1996). For many MHC/peptide complexes the repertoire of TCRs that achieve this affinity is large. In the case of the MHC/peptide ligand studied in detail in this investigation, A^b/PCC, this is true. T cells specific for this complex, positively selected by A^b bound to many different mouse peptides, could be generated with ease from normal mice, and they bore many different TCRs, albeit limited to some degree in the Vβ/Vα combinations they bore. Given this, it is perhaps not surprising that A^b bound to a single peptide could select T cells specific for A^b/PCC and, indeed, for the same MHC bound to all of the peptides we tested.

The collection of TCRs selected by the A^b/single peptide combination was smaller and different, however, from that selected by A^b bound to many peptides. Few T cells specific for A^b/PCC could be harvested from A^bEpIi^- mice, and they bore TCRs that were related but not identical to those found on A^b/PCC–specific T cells from A^bwt animals. Three possible factors may explain these observations. First, productive engagement of A^b/PCC must require some characteristics of the TCR. Vβs 8.1, 8.2 and 14, for example, are acceptable, whereas other Vβs such as 2 and 4 are probably precluded because the amino acids of their CDR1 and CDR2 regions are incompatible with reaction with A^b/PCC. Probably

the similarities between A^b/PCC–specific TCRs selected on A^bwt and A^bEp were attributable in part to this requirement.

Second, the TCR repertoires of $CD4^+$ T cells in A^bwt and A^bEpli$^-$ mice are not identical because cells in the former animals are tolerant to A^b bound to many mouse peptides, whereas cells in the latter are tolerant only to A^bEp. This difference might affect the results reported here, since only one of the TCRs in this report came from A^bEpli$^-$ mice that were tolerant to A^bwt. Perhaps this is why one out of the five A^b/PCC–specific hybridomas from A^bEpli$^-$ mice bore Vβ12, whereas this Vβ was not found on the more than 1 of 100 A^b/PCC–specific hybridomas analyzed from A^bwt-containing animals. Possibly all of the Vβ12, including TCRs that can bind A^b/PCC, cross-react on A^b bound to some mouse peptide.

Finally, the combination of A^b and one peptide selects fewer T cells than A^b bound to many peptides. This was first demonstrated by the finding that fewer $CD4^+$ T cells were selected in A^bEpli$^-$ animals than in A^bwt or A^bwtli$^-$ animals (Ignatowicz et al., 1996) and is illustrated in this report by the finding that the TCR repertoire of A^bEpli$^-$ animals for A^b/PCC does not include T cells bearing Vβs 6, 8.2, and 14. Since only six independent A^b/PCC–specific T cells were obtained from A^bEpli$^-$ animals, it was possible that this absence was due to inadequate sampling of the A^bEpli$^-$ TCR repertoire. However, the six T cell hybridomas obtained were all that were derived from four different priming attempts. Therefore it is more likely that the difference is due to the considerably smaller TCR repertoire in A^bEpli$^-$ animals than in A^bwt mice and suggests a failure of the A^bEp combination to select A^b/PCC–specific T cells bearing the absent Vβs.

Since none of the A^b/PCC–specific TCRs we tested had exactly the same structure as any of the others, we probably did not sample all possible A^b/PCC reactive TCRs from A^bwt animals or even from A^bEpli$^-$ mice. Thus, we cannot conclude that T cells bearing exactly the same TCR can or cannot be selected on A^bEp and A^bwt, nor can we conclude that the repertoire of TCRs selected by A^bEp is included within that selected by A^bwt. However, T cells specific for A^b/PCC and bearing Vβ13 were much more frequent in B10.A(5R) mice and A^bEpli$^-$ animals (if one of six hybridomas is representative) than they were in A^bwt mice. The Ep peptide is not bound to A^b in A^bwt animals, although it is in B10.A(5R) animals. Therefore these results may be indicative of preferential selection of A^b/PCC–specific T cells bearing Vβ13 on A^bEp rather than A^bwt, and therefore the TCR repertoire selected on A^bEp may not be completely included within that selected by A^bwt.

Experimental Procedures

Mice

The generation of mice expressing A^bEp and lacking other class II β chains and Ii has been described elsewhere (Ignatowicz et al., 1996) They and mice lacking all class II and Ii were bred in the Animal Care Facility at the National Jewish Medical and Research Center (NJMRC). C57Bl/10 (A^bwt), C57Bl/6, and B10.A(5R) animals were purchased from the Jackson Laboratory.

To make chimeric mice, 6- to 12-week-old A^bEpli$^-$ animals were given 950 rad and immediately reconstituted with 5×10^6 fetal liver cells from C57Bl/6 fetuses at day 16 gestational age. Animals were allowed to reconstitute for at least 6 weeks before use.

Direct Measurement of Peptide Binding to A^b

Peptides related to Ep were synthesized by the Molecular Resource Center at NJMRC. Spleen cells from A^bwtIi$^-$ mice were resuspended in complete culture medium at 10^6/ml with various concentrations of the Ep-related peptides and cultured for 8 hours at 37°C. Cells were then washed and stained with biotinylated anti-A^b/Ep (Yae) (Murphy et al., 1989; Rudensky et al., 1991b) as previously described (Scherer et al., 1995; Ignatowicz et al., 1996). Staining was measured on a FACScan cytofluorometer (Becton Dickinson). Results were expressed as the median fluorescence intensity of the stained peak of cells measured in arbitrary units.

Indirect Measurement of Peptide Binding to A^b

A collection of peptides known to bind A^b (Suzuki and Schwartz, 1986; Rudensky et al., 1991a) were synthesized by the Molecular Resource Center at NJMRC. Spleen cells from A^bwtIi$^-$ mice were incubated at 10^6 cells/ml in complete culture medium at 37°C for 8 hours with a limiting concentration (10 μg/ml) of Eα 52–66 and various concentrations of other peptides thought to bind A^b. The cells were then washed, and the amount of Eα 52–66 bound was measured as described above for the direct binding assay. Results were expressed as the median intensity of staining with anti-A^b/Ep.

T Cell Priming

$CD4^+$ T cells were purified from A^bEpli$^-$ mice and cultured in Click's medium, 1% normal mouse serum with 3000 rad–irradiated spleen cells from A^bwtIi$^-$ mice as presenting cells, and 100 mg/ml of the indicated peptide. Three to four days later, T cell blasts were harvested and fused to BW$\alpha^-\beta^-$ (White et al., 1989). Hybrids were screened for their ability to respond well to A^bwtIi$^-$ spleen cells plus 100 mg/ml of the relevant peptide and poorly, if at all, to A^bwtIi$^-$ spleen cells in the absence of peptide (Kappler et al., 1981).

Antigen-specific T cells were obtained from normal C57Bl/6 (A^bwt) or B10.A(5R) mice or A^bEpli$^-$ chimeras after in vivo immunization in the base of the tail (Kappler et al., 1981). One week later cells were harvested from the draining lymph nodes and cultured in vitro for 4 days with the relevant antigen, followed by 3 days of expansion with interleukin-2. The activated T cells were fused to BW$\alpha^-\beta^-$ to create T cell hybridomas, and the hybridomas were assayed for antigen reactivity as previously described (Kappler et al., 1981; White et al., 1989).

Analysis of TCRs on T Cell Hybridomas

Vβ and Vα use was screened by flow cytometric analysis of the hybridomas after staining with all the available anti-mouse Vα and Vβ reagents (Scherer et al., 1995). Further analysis was performed on cDNA made from the hybridomas. In brief, RNA was made from the hybridomas using the Ultraspec RNA preparation kit (Biotecx Laboratories, Houston, TX). This was converted to cDNA by polymerase chain reaction (PCR) with random hexanucleotide primers. cDNA that included Vβ8 sequences was amplified by PCR using a positive-strand oligonucleotide built to match members of the Vβ8 family and a negative-strand oligonucleotide built to match the 5′ end of Cβ. These oligonucleotides were used as primers for sequencing reactions with the amplified cDNAs using the ABI Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit. Vα use by the hybridomas was established by PCRs using a set of Vα positive-strand primers built to match all of the known Vα sequences (Arden et al., 1995) and a negative-strand oligonucleotide built to match the 5′ end of Cα. Amplified cDNAs from PCRs that yielded detectable bands were sequenced using the same oligonucleotides described above.

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